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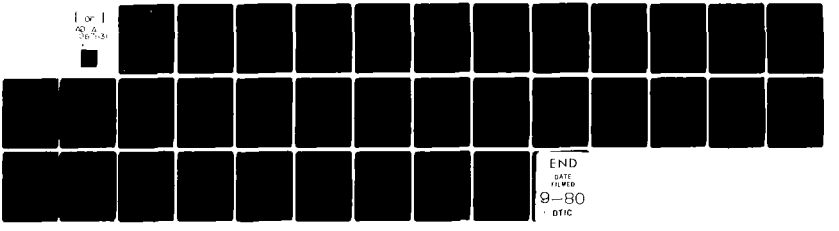

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# Mechanisms of Oxygen Toxicity at the Cellular Level

By

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PROJECT TITLE:

"MECHANISMS OF OXYGEN TOXICITY AT THE CELLULAR LEVEL"

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This report consists of the following: (I) Summary of Accomplishments, (II) Detailed Accomplishments and III Publications list.

## I. SUMMARY OF ACCOMPLISHMENTS

Significant progress occurred in four areas: (1) Effect of hyperoxia on reverse glycolysis: We conclude that glucose protects Escherichia coli against hyperoxia better than other intermediates later in glycolysis and in the Krebs cycle but not because of a specific inhibition of reverse glycolysis. There was no significant impairment of the three enzymes of reverse glycolysis: fructose-1,6-diphosphatase, phosphoenolpyruvate synthase, or phosphoenolpyruvate carboxykinase in cells poisoned by hyperoxia.

Indirect evidence also suggested that protection by glucose was not due exclusively to either an elevated synthesis of superoxide dismutase, or a lesser production of toxic oxygen radicals by cells grown with glucose. (2) Effect of hyperoxia on synthesis of RNA, DNA and pyridine nucleotide coenzymes: Evidence was obtained for direct inhibition of RNA synthesis in cells poisoned by hyperoxia, while protein synthesis was not directly affected. Protein synthesis is indirectly reduced because of impaired synthesis of specific amino acids as previously reported. DNA synthesis is impaired but it is not proven whether this is direct or indirect. NAD and NADH were shown to be severely decreased in cells during poisoning by hyperoxia. This decrease was prevented by including niacin, but not quinolinate in the medium which is consistent with poisoning of quinolinate phosphoribosyltransferase as the site of poisoning, as previously reported. PRPP (an intermediate in pyridine nucleotide coenyzme synthesis) does not protect against oxygen poisoning, PRPP synthetase is not affected by hyperoxia, but PRPP does accumulate in oxygen poisoned cells. Accumulation of PRPP is in agreement with inhibition by hyperoxia of quinolinate phosphoribosyltransferase, which uses PRPP as a substrate. (3) Relationship of thiamine to oxygen toxicity. Thiamine is protective for E coli exposed to hyperoxia. Oxygen exposure appears to poison an enzyme in the pathway for synthesis of hydroxymethyl pyrimidine, required for thiamine synthesis. Other enzymes in the pathway are not affected.

## II. DETAILED ACCOMPLISHMENTS

Two major areas of research were designated for the current contract year: (1) The hyperbaric oxygen sensitivities of two enzymes of reverse glycolysis, and (2) the effects of hyperoxia on RNA synthesis. Progress in these areas will be separately described in detail. In addition related experiments have been done to further extend our previous studies of oxygen poisoning of NAD biosynthesis and its relationship to item (2). In addition,

we have completed a proposed study of effects of thiamine which had been delayed bacause of unavailability of thiamine biosynthetic intermediates.

(1) THE HYPERBARIC OXYGEN SENSITIVITIES OF ENZYMES OF REVERSE GLYCOLYSIS.

Introduction: Previous research had indicated that oxygen poisoning of fructose-1,6-diphosphatase was minimal and could not account for the observed fact that Escherichia coli grew better in hyperoxia with glucose present than with other carbon and energy sources which enter metabolism further along in the Embden Meyerhof and Krebs cycle. We proposed to further study this phenomenon by direct analysis of other enzymes required for reverse glycolysis [i.e., synthesis of glucose from compounds more distal in the pathways].

## MATERIALS AND METHODS

Culture Conditions:

Wild-type Escherichia coli K12, obtained from the E. coli Genetic Stock Center at Yale University School of Medicine, was used in all experiments. The organism was grown in a minimal basal salts medium (Brunker, R.L. and Brown, O.R. Microbios. 4:192-203, 1971) with the indicated supplements of carbohydrate, amino acids and/or vitamins. To insure that the cells were able to transport the supplemented carbohydrate, they were grown with the compound as a sole carbon source for several days with repeated transfers to like medium. Before an experiment, these cells were grown in the same medium plus 20 amino acids for about 24 hours, with repeated transfers. All experiments were performed with logarithmically growing cells.

Cultures were grown at 37°C with aeration by stirring or shaking.

Growth rate was determined by measuring the increase with time in absorbance at 500 nm with a Gilford spectrophotometer. Cultures with absorbances greater than 0.8 were diluted before precise determinations of absorbance.

Preparation of Cell Extracts:

Cultures to be prepared for fructose-1,6-diphosphatase (FDPase) assay were grown in the minimal basal salts medium, 27.7 mM pyruvate, and 20 common amino acids at 0.65 mM each (Boehme, D.E., Vincent, K. and Brown, O.R., Nature 262:418-420, 1976). Cultures to be prepared for phosphenolpyruvate (PEP) synthease and PEP carboxykinase assays were grown in the minimal basal salts medium with 27.7 mM lactate, 20 amino acids at 0.65 mM each, 0.2 mM niacin, and 0.1 mM thiamine. Cultures, in exponential growth at 37°C in a stirred vessel, were pressurized with 4.2 atm of oxygen (gas phase: 1 atm air plus 4 atm oxygen). The vessel was fitted with a separate compartment which contained chloramphenicol. After 60 minutes of exposure to hyperbaric oxygen, the chloramphenicol (final concentration, 150 μg/ml) was tipped into the culture, the vessel was decompressed rapidly, and the culture was poured over cracked ice. The cells were removed by centrifugation at 4°C, washed one time, and resuspended to a concentration of approximately 0.7 grams wet weight of cells in 3 ml. The washing and resuspension buffer with either 0.04 M triethanolamine, pH 7.5, containing 150 μg chloramphenicol/ml for FDPase assays, or 0.02 M Tris-HCl, pH 7.5, with 1 mM $MgCl_2$, and 150 μg chloramphenicol/ml for PEP synthase and PEP carboxykinase assays. The cells were disrupted by sonication at 4°C for 3 min at 90% power with a Bronwill sonic probe operated for 30 sec intervals separated by intervals for cooling. The disrupted cell suspensions were

centrifuged at 4°C for 20 min at 18,000 x g and the supernatants were used for enzyme assays. The extracts for FDPase and PEP carboxykinase assays were stored at 15-20°C. The extracts were neither purified nor dialyzed since the objective was to detect potential oxygen inactivation of specific enzymes and it was desirable to minimize manipulations which might differentially affect enzyme specific activities in control and experimental cultures. Protein in the extracts was measured by a modification of the Lowry method as described by Hartree (Hartree, 1972).

It has been established previously that the primary effect of hyperbaric oxygen exposure is bacteriostatic, not bactericidal (Brown, O.R., et. al., J. Lipid Research 12:692-698, 1971), and that growth inhibition results from hyperoxia, not from increased pressure per se (Brown, O.R., Radiation Research 50:309-318, 1972).

Enzyme Assays:

The specific activities of three enzymes of reverse glycolysis were measured. Fructose-1,6-diphosphatase (E.C. 3.1.3.11) was assayed by the method of Pontremoli (Methods in Enzymology 9:625). The reaction mixture contained 40 mM glycine-NaOH buffer, pH 9.4, 1 mM $MnCl_2$, 10 mM fructose-1,6-diphosphate, and up to 2 mg extract protein. The amount of phosphate liberated was determined according to the method of Fiske and Subbarow (Methods in Enzymology 3:843).

Phosphoenolpyruvate synthase activity was measured by the method of Benziman (Methods in Enzymology 42:192). The reaction mixture contained 100 mM Tris-$H_2SO_4$, pH 8.2, 10 mM $MgCl_2$, 10 mM potassium phosphate, pH 8.2, 20 mM sodium succinate, pH 8.2, 2 units pyrophosphatase/ml, 5 mM ATP, 2 mM pyruvate, and up to 1 mg extract protein/ml. The phosphoenolpyruvate produced was measured spectrophotometrically by the oxidation

of NADH in an assay mixture of 100 mM Tris-$H_2SO_4$, pH 8.2, 5 mM $MgCl_2$, 50 mM KCl, 0.5 mM ADP, 0.5 mM NADH, 3.6 units of lactate dehydrogenase/ml, and 3.0 units of pyruvate kinase/ml.

The activity of phosphenolpyruvate carboxykinase (E.C. 4.1.1.49) was determined according to the method of Utter and Kurahashi (J.B.C. 207:787, 1954) as modified by Wright and Sanwal (J.B.C. 244:1838, 1969). The assay mixture contained 100 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 3.5 mM ATP, 20 mM $NaH^{14}CO_3$ (5-7.5 μCi/ml), 20 mM oxaloacetate, and 0.25-0.75 mg extract protein/ml.

Chemicals:

Radioactive sodium bicarbonate was purchased from Amersham-Searle. All other chemicals were obtained from Sigma Chemical Co. or Fisher Scientific Co.

Results and Discussion:

Previous work from this laboratory with another strain of E. coli showed that different carbon sources provided varying amounts of protection during hyperoxia (Brown, O.R., Boehme, D., and Yein, F., Microbios. 23:175-192, 1979). As shown in Table 1, there is a similar effect of different carbohydrates on the growth of E. coli K12 during 1 hour of hyperbaric oxygen exposure.

Generation times with a gas phase of 1 atm air were calculated over the interval of 30 minutes prior to hyperoxia from the slope of best fit relating the logarithm of absorbance at 500 nm to minutes of incubation. Generation times in hyperoxia were extrapolated from changes in the logarithm of absorbance during 1 hour in high pressure oxygen. As

would be expected, the generation time in air is different for different carbon sources. Since the generation time during hyperoxia is very likely dependent upon the generation time in air, the protection afforded by a particular compound must be expressed in terms of the air generation time. Therefore, the percentage protection has been calculated as the number of generations per hour in air, expressed as a percentage. This number, then, adjusts the data for specific effects of individual compounds as they affect growth rates in air, with 0% protection indicating that no growth occurred during hyperoxia and 100% protection indicating that the same amount of growth occurred in hyperoxia as occurred in air. Values are means plus or minus 1 standard deviation for 4 determinations, except for media with glucose where 20 determinations were averaged, and media with no carbohydrate added where 8 determinations were averaged. Glucose allows for more growth, i.e, provides more protection, during hyperoxia than any of the other carbon source tested. The other compounds provide an amount of protection seemingly dependent on their distance from glucose in the glycolytic pathway; those carbohydrates closest to glucose giving the most protection. Protection by fructose-1,6-diphosphate appears to be an anomaly since it allows very little growth during hyperoxia yet it is formed between fructose-6-phosphate and the triose phosphates (the entry point for glycerol) in glycolysis. E. coli K12, however, was unable to use fructose-1,6-diphosphate as a sole carbon source (Fig. 1), probably because of inability to transport the diphosphate, and any protection in excess of that given by the amino acids alone is most likely due to breakdown of the compound to a monophosphate or fructose.

Table 2 shows that the activities of FDPase, PEP synthase, and PEP

carboxykinase are not greatly reduced by in vivo exposure to high pressure oxygen. The activities of FDPase and PEP synthase in cells exposed to hyperoxia for 1 hour are significantly different from those in air-grown cells at the $P \leq 0.01$ and 0.05 levels, respectively, but the magnitude of the differences preclude biological significance.

Although there appears to be no effect of hyperoxia on reverse glycolysis, there is still more growth during oxygen exposure when glucose is the carbon source. There are two alternate explanations. One is that when the cells grow on glucose, they produce more superoxide dismutase. Another is that some compounds other than glucose, such as pyruvate, cause the cell to produce more superoxide or other toxic radicals, which overcome the cell's ability to dispose of them. To test these two hypotheses, E. coli K12 was grown in a mixture of glucose and pyruvate (27.7 mM each) and exposed to 4.2 atm of $O_2$ for 1 hour. If more superoxide dismutase is produced during growth on glucose, then the glucose and pyruvate mixture should protect much like glucose. If more toxic radicals are produced during growth on pyruvate, then the mixture should allow about as much growth as pyruvate alone. Glucose and pyruvate together afforded protection approximately halfway between that of glucose and pyruvate separately (Table 1). Therefore, neither hypothesis can be exclusively correct.

TABLE 1

EFFECTS OF VARIOUS COMPOUNDS ON THE GROWTH OF *ESCHERICHIA COLI* K12 IN 4.2 ATM HYPERBARIC OXYGEN
(1 hour exposure)

| Supplemented Carbohydrate[a] | Ability to Serve as Sole Carbon Source | Generation Time (min)[b] AIR | Generation Time (min)[b] HPO | Percentage Protection[c] | # Generations/hr in Hyperoxia | Normalized Ratio[d] |
|---|---|---|---|---|---|---|
| None | Not Applicable | 35.8±1.8 | 140 ±12.8 | 25.9±3.4 | 0.435±0.042 | 0.35 |
| Glucose | Yes | 37.4±2.5 | 51.0±4.9 | 73.9±7.9 | 1.19 ±0.114 | 1.00 |
| Fructose-6-Phosphate | Yes | 28.9±1.6 | 53.2±3.4 | 55.1±4.5 | 1.15±0.068 | 0.74 |
| Fructose-1,6-Diphosphate | No[e] | 40.7±5.3 | 128 ±9.5 | 33.3±2.4 | 0.468±0.033 | 0.43 |
| Glycerol | Yes | 34.8±1.1 | 64.6±3.4 | 54.1±4.6 | 0.936±0.050 | 0.73 |
| Pyruvate | Yes | 36.7±1.2 | 99.4±1.3 | 37.0±1.2 | 0.608±0.008 | 0.50 |
| Lactate | Yes | 36.9±1.2 | 86.3±3.5 | 42.9±1.9 | 0.702±0.030 | 0.58 |
| Glucose + Pyruvate | Yes | 30.4±1.8 | 57.8±4.2 | 51.2±5.0 | 1.02±0.044 | 0.72 |

Footnotes to Table 1:

[a]A minimal basal salts medium (Brunker and Brown, Microbios 4:192, 1971) was supplemented with 20 common amino acids at 0.65 mM each (Boehme, et al., Nature 262:418, 1976) and the indicated carbohydrate at 27.7 mM. In the glucose plus pyruvate mixture, each was present at 27.7 mM. NOTE: Some of these data was previously reported (Report #32, Jan. 1, 1978) but are repeated for completeness and continuity.

[b]Cultures were adapted to growth in the individual test media with air as the gas phase and grown in small vials with stirring in a vessel which could be pressurized. The vessel was incubated in a constant temperature bath at 37°C. During exponential growth at an absorbance of 0.1 to 0.2 at 500 nm wavelength, the cultures were subjected to a gas phase of 1 atm of air plus 4 atm oxygen (HPO) by pressurization with pure oxygen. After 1 hr, the vessel was decompressed. All samples for absorbance measurements were collected in tubes chilled in ice and measured immediately with a Gilford spectrophotometer. Generation times with a gas phase of 1 atm of air were calculated over an interval of 30 min from the slope of best fit relating four determinations of the logarithm of absorbance at 500 nm to minutes of incubation; generation times in HPO were extrapolated from changes in the logarithm of absorbance during 1 hr in HPO. Values are averages ± 1 standard deviation for 4 determinations, except 8 determinations were averaged for media with glucose + pyruvate and with no carbohydrate added, and 20 determinations were averaged for media with glucose.

[c](Number of generations per hr in HPO/number of generation per hr in air) x 100.

[d](Percentage protection in a given medium)/(Percentage protection in glucose medium). This adjusts the data for specific effects of individual compounds as they affect growth rates in air and normalizes all data with respect to data for medium with glucose (the compound which permitted most growth in HPO).

[e]Inability to utilize fructose-1,6-diphosphate, supplied as the sole carbon source (see Fig. 1), accounts for the lack of protection in hyperoxia when supplied as a supplement in complex medium.

TABLE 2

EFFECTS ON ENZYMES OF REVERSE GLYCOLYSIS OF EXPOSURE OF *E. COLI* K12 TO 4.2 ATM OF OXYGEN FOR 1 HOUR

| Enzyme | Specific Activity[a] | |
|---|---|---|
| | AIR | 1 hour HPO |
| Fructose-1,6-Diphosphatase[b] | 0.172±0.030 (3,36) | 0.145±0.031 (2,23)[e] |
| Phosphoenolpyruvate Synthase[c] | 0.033±0.010 (5,42) | 0.041±0.009 (2,12)[f] |
| Phosphoenolpyruvate Carboxykinase[d] | 7.45±0.60 (2,9) | 7.35±1.13 (2,9) |

[a]Mean ± standard deviation (# experiments, # assays)

[b]Specific activity reported in micromoles phosphate liberated/minute/mg protein.

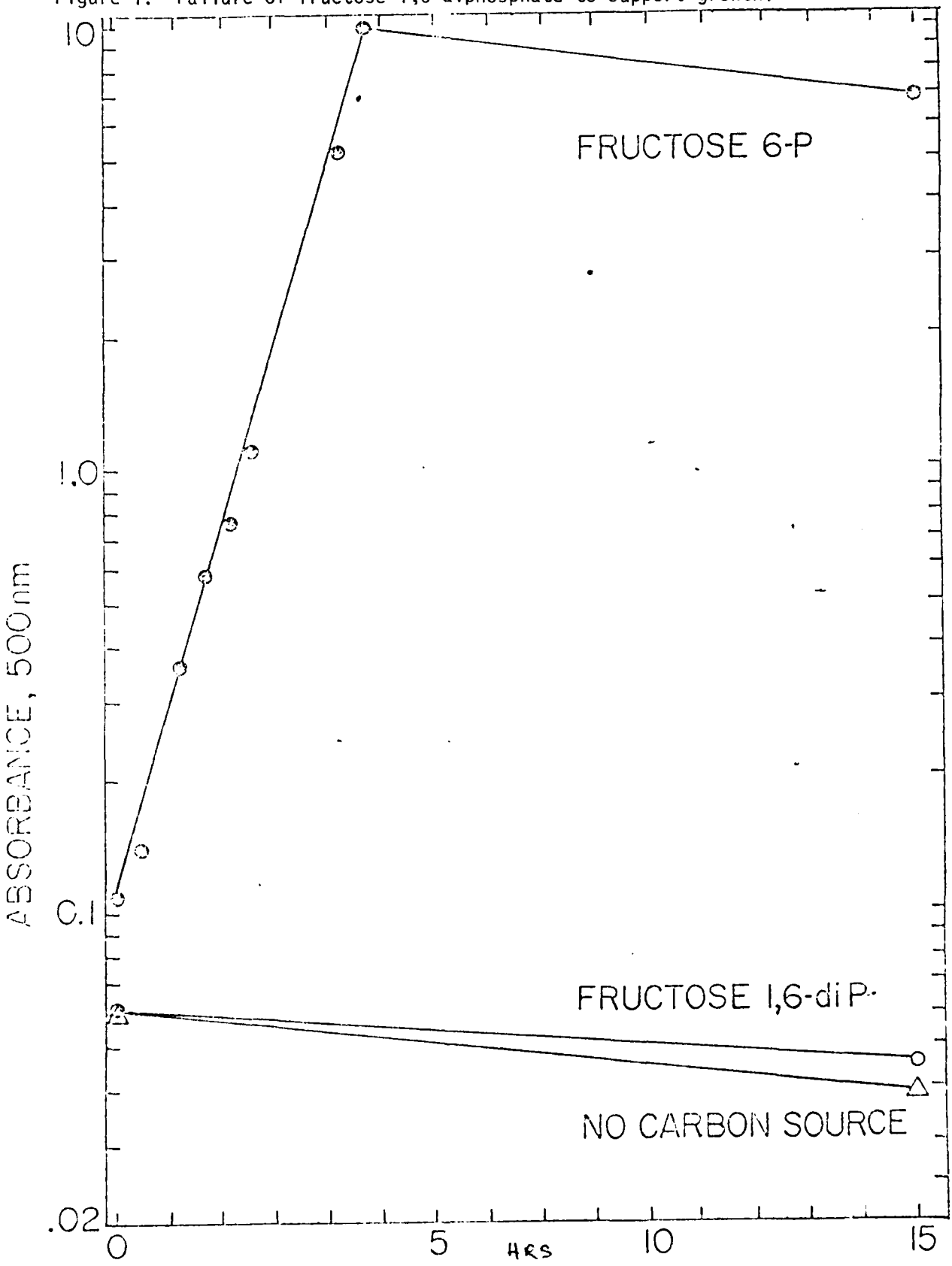
[c]Specific activity reported in micromoles PEP formed/minute/mg protein.

[d]Specific activity reported in nanomoles $CO_2$ exchanged/minute/mg protein.

[e]Significantly different from air control activity at $P \leq 0.01$, using Student's t test.

[f]Significantly different from air control activity at $P \leq 0.05$, using Student's t test.

Figure 1. Failure of fructose-1,6-diphosphate to support growth.



## (2-A) THE EFFECT OF HYPERBARIC OXYGEN ON RNA SYNTHESIS

Introduction: This research was conducted to extend observations previously made regarding possible inhibition of RNA synthesis by hyperoxia ["Bacterial sites of oxygen toxicity potentially common to red cells and erythropoiesis," O.R. Brown, F. Yein, and D. Boehme, (1978) in: The Red Cell, ed. by G. Brewer, Alan R. Liss Publisher, N.Y., pp. 701-704]. For comparative purposes, RNA, DNA and protein synthesis and total growth, as measured by absorbance changes were measured in cells growing in air and after exposure to 1 atm of air plus 1 atm of oxygen (HPO).

Methods: E. coli NF161 (stringent) and NF162 (relaxed) were maintained in MBS plus 27 mM glucose plus 0.65 mM arginine and 0.65 mM methionine. Before experiments, cultures were grown at least 24 hrs with several transfers in the same medium supplemented with 20 amino acids (each at 0.65 mM) and 0.2 mM niacin and 0.1 mM thiamine. For experiments, exponentially growing cells were transferred to the same medium except that it contained 14 mM radioactive glucose (0.056 μCi of [$^{14}$C]-U-glucose/μmole glucose). The organism was grown for 4 or 5 generations to approach 100% labeling of RNA, DNA and protein. Part of the culture was then exposed to a mixture of 1 atm of air plus 4 atm of oxygen for 30 min. Triplicate samples at time zero and 30 min were taken from both cultures. The cultures were extracted and the RNA, DNA and protein were separated as described by Kennell ["Use of filters to separate radioactivity in RNA, DNA, and protein (1968), D. Kennell, In: Methods in Enzymology, ed. by L. Grossman, Vol. 12, pp. 686-693]. The radioactivity

in each fraction was determined by scintillation spectrometry and the number of generations or fraction thereof was determined from the relationship: $N_t=N_0 2^x$, where $N_t$ and $N_0$ = radioactivity at 30 min and time zero, respectively, and x = no. of generations.

Phosphoribosylpyrophosphate synthetase was measured by a modification of the two part assay of Kornberg et. al (J.B.C. 215:389, 1955). The reaction for part one (1.1 ml) contained: • 66 µmole potassium phosphate buffer, pH 7.4 2 µmole ATP, 4 µmole ribose-5-phospate, 10 µmole $MgCl_2$, 10 µmole dithiothreitol and 10-20 µl of crude enzyme extract (0.2 to 0.4 mg protein). A reaction with ATP and ribose-5-phosphate omitted was used as the blank. Following incubation for 10 min at 37°C, the reaction was stopped by heating at 100°C for 30 sec and cooling immediately in an ice-acetone bath. Precipitated protein was removed by centrifugation at 10,000 for 10 min. The reaction for part two (0.55 ml) contained: 11 µmoles tris-HCl, pH 8.0, 0.68 units of orotidinepyrophosphorylase-decarboxylase, 1.1 µmole $MgCl_2$, 0.3 µmoles [carboxyl-$^{14}$C]-orotic acid (0.5 µCi/µmole) and 100 to 300 µl of reaction supernatant. The reaction was started by addition of radioactive orotate and after 60 min at 37°C was terminated by injection of 0.1 ml 5N $H_2SO_4$ through a rubber stopper. $CO_2$ was trapped and radioactivity was determined as described for the quinolinate phosphoribosyl transferase assay. Tests were done to arrive at the above substrate and enzyme concentrations, and incubation durations as within the linear limits of the assay. One mU of enzyme produced one µmole of $CO_2$ per hour.

The intracellular pool of phosphoribosylpyrophosphate was determined using the reaction for part two of the assay (J.B.C. 215:309, 1955). Crude extracts was heated at 100°C for 60 sec and immediatley cooled in an ice-acetone bath. Protein was removed by centrifugation and 0.2

ml of the supernatant was assayed as described above except that 0.1 mM [carboxyl-$^{14}$C]-orotic acid (5 μCi/μmole) was used.

Results and Discussions

The results are similar to data previously obtained using other strains but have been extended. There were definite impairments in synthesis of both RNA and DNA which are not explained by the known induction of stringency by hyperoxia (Table 3). Both stringent and relaxed strains showed similar reductions in growth and synthesis in hyperoxia compared to air (Table 4). The reductions in protein synthesis and in growth, as measured by absorbance change, were small while the reductions in synthesis of RNA and DNA were comparatively large (Table 4). Conditions were chosen so that stringency was prevented from being expressed two ways: (a) by use of the relaxed strain which does not possess the stringency gene and (b) by inclusion of 20 amino acids in the culture medium with the stringent strain. Clearly, then, the inhibition of RNA and DNA synthesis seen in Tables 3 and 4 cannot be attributable to inhibition by guanine tetraphosphate (ppGpp) as a result of induction of stringency by hyperoxia.

It appears likely that a single site, or multiple sites, in biosynthesis of RNA and DNA, are sensitive to inactivation in cells exposed to hyperoxia. We previously had speculated that the inhibition of DNA synthesis was indirect, being due to failure of protein synthesis. The evidence for this was an apparent correlation between the amount of protein and DNA synthesis and the fact that synthesis of "initiator" protein is required prior to each new round of DNA synthesis following completion of the chromosome in E. coli. However, in previous experiments,

the cells were incubated for 1 hour in hyperoxia and the total amount of DNA synthesized was very similar to that obtained during the experiments of Table 3, where the incubation in hyperoxia was for only 30 min. Most of the growth and protein synthesis which occurred during the 1 hour exposures to hyperoxia, therefore, probably occurred during the first 30 min. Note that there was very little inhibition of protein synthesis in the 30 min in hyperoxia while DNA synthesis was inhibited (Table 3). This evidence is not in contradiction to our previous data, but expands our view and indicates for the first time to us that hyperoxia is producing, by direct means, an inhibition of DNA synthesis.

In this regard, an interesting paper has just come to our attention: "Human Amidophosphoribosyltransferase: an Oxygen-Sensitive Iron-Sulfur Protein", M. Itakura and E.W. Holmes. J.B.C. 254:333-338 (1979). This enzyme was found to be extremely sensitive in vitro to molecular oxygen. It catalyzes the first and probably rate-limiting step in de novo purine biosynthesis. If this enzyme is inactivated in vivo, this could cause the observed decrease in synthesis of RNA and DNA. Since the same pathway, containing this enzyme is universally used for purine nucleotide synthesis, the significance of this site for biological oxygen toxicity is apparent to us, although no mention of this possible relationship was made by Itakura and Holmes.

Preliminary data regarding effects of hyperoxia on phosphoribosyl-pyrophosphate (PRPP) synthetase had shown conflicting results in that PRPP synthetase appeared to be partially inactivated in hyperbaric oxygen-exposed cells, but addition of PRPP to culture medium did not provide protection. As a result of much continued effort we have ascertained that the previous measurements showing decreased PRPP synthetase activity were in error because of the following facts. The measured PRPP synthetase activity

is strongly affected by the amount of cell-free extract in the assay, i.e., the activity is proportional to amounts of protein only at concentrations below about 0.4 mg protein of crude extract. Higher concentrations show less total activity, thus specific activity is severaly reduced. In our previous preliminary work [Brown, O.R., Yein, F., and Boehme, D. In: The Red Cell, G.J. Brewer, ed. (1978) pp. 701-714, Alan R. Liss, N.Y.] we had compared assays of air-grown control cells using small amounts of protein with assays of oxygen-exposed cells using a range of protein, up to 14 mg per assay. This was done deliberately in an attempt to obtain more activity for more precise determination in case the enzymes were severely inactivated. In these studies we accepted the results of the assays with large amounts of protein since we had not done controls which would have revealed the unexpected severe departure from linearity.

The results are clear now and consistent. PRPP does not protect cells agaonst hyperoxia, PRPP content of oxygen-poisoned cells is even higher (Table 5) than normal (not lower), and PRPP synthetase activity is not changed (Table 5). The reason for the increased intracellular PRPP content in oxygen-poisoned cells can be explained by the impairment in activity in quinolinate phosphoribosyltransferase we have found in cells poisoned by hyperoxia. Increased PRPP results from accumulation of PRPP since it is a substrate for the poisoned enzyme. It is also interesting to note that the amidophosphoribosyltransferase, previously discussed as a possible site of impairment of RNA and DNA synthesis in hyperoxia, also uses PRPP as a substrate.

Indeed, we have also found PRPP content to be elevated in mammalian cells exposed to hyperoxia, in related studies not funded by this contract, which provides further confirmation of these mechanisms.

TABLE 3

Effects of hyperoxia on growth and synthesis of stringent (NF161) and relaxed (NF162) strains of Escherichia coli[a]

| Strain | Gas phase | No. of generations in 30 min[b] | | | |
|---|---|---|---|---|---|
| | | RNA | DNA | Protein | Absorbance |
| Stringent | Air | 0.84±0.07 | 0.85±0.10 | 0.92±0.01 | 1.13±0.08 |
| | HPO | 0.41±0.07 | 0.41±0.24 | 0.76±0.14 | 0.94±0.03 |
| Relaxed | Air | 0.90±0.11 | 0.96±0.15 | 0.97±0.20 | 1.10±0.03 |
| | HPO | 0.51±0.10 | 0.57±0.09 | 0.82±0.21 | 0.97±0.07 |

[a]Cells in exponential growth in minimal medium supplemented with 0.27 mM glucose, 0.2 mM niacin, 0.1 mM thiamine, and 20 amino acids (each at 0.65 mM) were grown at 37°C with stirring for 30 min with a gas phase of air at 1 atm or 1 atm of air plus 4 atm of oxygen (HPO). Pressure, per se at 4 atm produces no effect.

[b]Averages ± 1 S.D. for 9 determinations from 3 experiments, except absorbance where single determinations were made from each of 3 experiments. The number of generations produced in 30 min was calculated from the change in absorbance or in radioactivity in the RNA, DNA and protein after separation.

TABLE 4

Impairment by hyperoxia on growth and synthesis in stringent (NF161) and relaxed (NF162) strains of Escherichia coli[a]

| Strain | Percentage of Air Control Synthesis | | | |
|---|---|---|---|---|
| | RNA | DNA | Protein | Absorbance |
| Stringent | 49 | 48 | 83 | 83 |
| Relaxed | 57 | 59 | 95 | 88 |

[a]Calculated from data of Table 3.

TABLE 5

Effects of hyperbaric oxygen on phosphoribosyl pyrophosphate (PRPP) synthetase and intracellular PRPP content of *E. coli*[a]

| Phase | PRPP Synthetase Specific Activity (MU/mg) | PRPP Pool (molecules/cell) |
|---|---|---|
| Air | 15.6±3.3 (2, 15) | $4.91 \pm 0.48 \times 10^3$ (3,12) |
| HPO | 15.1±2.0 (2,15) | $35.5 \pm 0.67 \times 10^3$ (3,12) |

[a] *Escherichia coli* K-12 was grown at 37°C in medium A (Table 1) which contained amino acids to prevent growth inhibition due to poisoning in the amino acid biosynthetic pathways (Boehme, D.E., Vincent, K., and Brown, O.R., Nature 262:418-420, 1976). HPO = 1 atm of air plus 4 atm of oxygen (4.2 atm $O_2$). The enzyme assay procedures and definitions of units of activity are given in Materials and Methods. Averages ± S.E.M. are shown with the number of experiments and total determinations in parenthesis.

[b] Significantly lower ($p \leq 0.001$) compred to air controls, using Student's t test.

(2-B) REDUCTION OF NAD BY HYPERBARIC OXYGEN

Introduction: We also have extended our study of the effect of hyperoxia on NAD synthesis. Although, as previously stated, these experiments were not specifically a part of our proposal during the course of the research they appeared to be vital and therefore were conducted.

Methods:

Escherichia coli strain K-12 obtained from the Genetic Stock Center of Yale University was grown and exposed to 4.2 atm of oxygen as previously described [Brown, O.R., and Yein, F., B.B.R.C. 85:1219 (1978)]. The cells were harvested through a cooling coil which reduced the temperatures of the culture from 37°C to 4°C within approximately one min. Cells were extracted in 0.5 N perchloric acid to preserve the oxidized coneyzmes and in 0.5 N KOH in ethanol:water (1:1 v/v) to preserve the reduced coenzymes [Klingenberg, M., Methods of Enzyme Analysis (1963) H.U. Bergmeyer, ed. Academic Press, N.Y. p. 528]. The coenzymes were assayed by a sensitive polarographic recycling method [Pender, S., Clark, J.B., and Greenbaum, A.L., Methods in Enzymology 18B, 20 (1971)].

Results and Discussion:

The concentration of NAD and NADH decreased dramatically during exposure to hyperbaric oxygen in medium without niacin or quinolinate (Table 6). NADP and NADPH, which were present initially in much smaller amounts, did not change significantly or increased transiently in hyperoxia (Table 6). The data suggest that the processes which convert NAD (the biosynthesized form of the coenzymes) into NADH, NADP, and NADPH are comparatively resistant to hyperbaric oxygen.

Addition of 0.2 mM niacin to the culture medium caused an approximate doubling of each coenzyme without affecting the oxidized/reduced ratios in cells growing with air as the gas phase (Table 6). Niacin prevented the decrease in coenzymes in hyperoxia (Table 6).

Without niacin or quinolinate, the oxidized to reduced ratios of NAD in hyperbaric oxygen at all time intervals were changed significantly toward a more reduced state and the ratio steadily decreased. A similar pattern was seen in cells grown in medium supplemented with quinolinate but the oxidized to reduced ratios changed less during the first hr and not at all thereafter in medium with niacin (Table 6).

It should be noted that the medium contained amino acids and thiamine, previously found to protect in hyperoxia. Amino acids must be present in order to observe the protective effects of niacin for growth in hyperoxia. The decreased growth rate with time in hyperoxia was correlated with the fall in intracellular NAD and NADH and the shift in oxidized to reduced ratios (Tables 6 and 7).

Even when the NAD concentration without niacin in the medium (Table 6), fell to 25% of normal at 1 hr and 7% at 2 hr, growth continued although the generation times were increased about 6-fold and 7-fold, respectively (Table 7). With niacin, growth rate still declined in hyperoxia but was increased less than 3-fold at 1 hr and was stabilized at about a 3 1/2-fold from 90 min onward (Table 7).

The decrease in coenzyme concentrations which occurred in Escherichia coli exposed to hyperbaric oxygen theoretically might result from inhibition of synthesis or increased breakdown. However, inhibition of synthesis is consistent with published observations that quinolinate phosphoribosyl

transferase, required for de novo synthesis of NAD, is poisoned in hyperoxia.

The failure of quinolinic acid (an intermediate in the biosynthesis of NAD prior to the oxygen-sensitive enzyme) to protect against growth inhibition (Table 7) or coenzyme decrease (Table 6) while niacin (which is below the oxygen-sensitive enzyme in the pathway) protected (Table 6 and 7) is also consistent with inhibition of coenzyme synthesis at this step.

(2-C) EFFECT OF PRPP, SOME VITAMINS, AND INTERMEDIATES ON GROWTH IN HYPERBARIC OXYGEN

Introduction: To complete some research on the interactions among amino acids, thiamine, niacin and PRPP, the experiments shown in Table 8 were done.

Methods: Escherichia coli strain K12 and the methods described for (1) The Hyperbaric Oxygen Sensitivities of Two Enzymes of Reverse Glycolysis, were used.

Results: Table 8 shows that addition of the 10 amino acids previously shown to be marginally beneficial for E. coli strain E26 also are slightly beneficial for strain K12 during the first 2 hrs, but that 10 amino acids (see footnote to Table 8, for the list) are most important. Niacin and thiamine are clearly beneficial, especially for longer exposures (2-4 hrs) but PRPP offers no advantage. Thiazole will not substitute for thiamine. This suggests that if hyperoxia prevents thiamine biosynthesis (which we have not proved) then the impairment is not in the synthesis of the thiazole ring.

TABLE 6. Decreases in coenzyme concentration and in growth rate of Escherichia coli during hyperbaric oxygen exposure, and protection against these decreases by niacin.

| Supplement | Min in HPO | Generation time (min) | NAD | NADH | NADP | NADPH | TOTAL | NAD/ NADH | NADP/ NADPH |
|---|---|---|---|---|---|---|---|---|---|
| | | | (Thousands of molecules per cell) | | | | | | |
| None | 0 | 26.8±0.9 | 918±161 | 665±92 | 44±9 | 65±8 | 1726±257 | 1.43±0.09 | 0.70±0.26 |
| None | 0-5 | - | 605±71[a] | 731±251 | 58±15 | 111±13[b] | 1505±341 | 0.87±0.19[b] | 0.52±0.08 |
| None | 0-15 | - | 464±111[b] | 589±69 | 94±22[b] | 99±11[b] | 1228±63[b] | 0.83±0.26[b] | 0.95±0.22 |
| None | 0-30 | - | 482±105[b] | 593±115 | 62±16 | 85±16 | 1204±221[a] | 0.81±0.07[c] | 0.76±0.28 |
| None | 0-60 | 65.0±3.2[c] | 228±42[c] | 335±75[b] | 32±2 | 60±11 | 637±154[b] | 0.68±0.3[c] | 0.53±0.06 |
| None | 0-120 | 134±99[a] | 58±57[c] | 113±71[c] | 25±15 | 52±28 | 260±227[c] | 0.44±0.18[c] | 0.49±0.15 |
| Niacin | 0 | 26.3±0.9 | 1826±161 | 1300±131 | 79±16 | 122±5 | 3327±298 | 141±0.07 | 0.65±0.13 |
| Niacin | 0-60 | 69.1±12.5[c] | 1589±175 | 1630±178 | 114±7[a] | 239±32[b] | 3573±118 | 0.99±0.19[a] | 0.48±0.06 |
| Niacin | 0-120 | 72.5±7.7[c] | 2059±449 | 2065±132[b] | 122±29 | 324±82[b] | 4474±646[a] | 0.99±0.17[a] | 0.38±0.05[a] |
| Quinolinate | 0 | 26.5±1.4 | 950±30 | 831±46 | 5219 | 68±6 | 1900±26 | 1.15±0.09 | 0.78±0.30 |
| Quinolinate | 0-60 | 64.9±4.8[c] | 235±105[b] | 319±70[b] | 37±19 | 59±7 | 650±176[c] | 0.71±0.18 | 0.64±0.22 |
| Quinolinate | 0-120 | 84.5±2.9[c] | 70±21[c] | 121±29[c] | 27±5 | 47±2 | 265±49[c] | 0.58±0.03[b] | 0.58±0.14 |

[a,b,c]Significantly different, respectively, at: $(0.01 < p \leq 0.05)$; $(0.001 < p \leq 0.01)$, $(p \leq 0.001)$ compared to the air control in medium with the same supplement.

Methods: Escherichia coli strain K-12 was grown at 37°C in glucose basal salts medium supplemented with 20 amino acids, each at 0.65 mM, thiamine, 0.1 mM and niacin (0.2 mM) or quinolinate (0.1 mM), where indicated. Cultures were pressurized with 4 atm of oxygen plus 1 atm of air (HPO) at an absorbance of approximately 2 at 500 nm wavelength where there are $1.56 \times 10^8$ cells per 1 absorbance in each of these media as determined by standard plate colony counting techniques. The relationship between colony counts and absorbance is linear up to 0.8; all measurements were made below this value by dilution as necessary. Cultures were stirred with a bar magnet via an external, coupled magnetic stirrer. The data are averages ± 1 S.D. for 3 to 5 experiments with triplicate determinations of coenzymes and single determinations of cultured absorbance for each experiment.

Table 7. The comparative increases in generation time during exposure of Escherichia coli to hyperbaric (4.2 atm) oxygen with and without niacin. The conditions were as described in Table 1 except that absorbance was continuously recorded using a Brinkman PC 1000 spectrophotometer with a probe sealed into the chamber.

| Time Intervals In Hyperoxia (min) | Generation Time (min) | |
|---|---|---|
| | With Niacin | Without Niacin |
| 60-70 | 73 | 158 |
| 70-80 | 73 | 161 |
| 80-90 | 91 | 167 |
| 90-100 | 98 | 174 |
| 100-110 | 96 | 182 |
| 110-120 | 99 | 189 |

TABLE 8

Comparative effects of several compounds for amelioration of hyperbaric oxygen toxicity for Escherichia coli

| Supplemented Compound[a] | Generation time (min)[b] | | |
|---|---|---|---|
| | 1 atm air | 4.2 atm Oxygen | |
| | | 0-2 hr | 2-4 hr |
| None | 41.2±6.3 | 951 | ∝ |
| 10AA+ Niacin | 34.1±1.4 | 90.4±2.1 | 530±58 |
| 10AA+ Niacin + Thiamine | 33.9±3.5 | 74.2±2.0 | 115±13 |
| 20 AA+ Niacin + Thiamine | 29.1±0.6 | 57.5±1.4 | 133±7.9 |
| 20 AA+ Niacin + Thiazole | 31.3±1.6 | 82.4±14.1 | 198±21 |
| 20 AA + Niacin | 32.3±2.3 | 71.0±2.2 | 216±26 |
| 20 AA+ Niacin + PRPP | 29.4 | 71.2 | 200 |

[a]Minimal based salts medium plus 27.7 mM glucose was supplemented with 0.65 mM each of 10 AA (asparagine, isoleucine, leucine, methionine, threonine, valine, phenylalanine, tryptophan, tyrosine, and cysteine) or 20 AA (the above 10 plus the other ten amino acids common to protein synthesis), niacin (0.2 mM), thiamine, (0.1 mM), PRPP (5 mM) or thiazole (5 mM).

[b]Averages ± 1 S.D. for 8 cultures from 2 experiments except when PRPP was used, only 1 experiment with 4 cultures was done.

## (2-D) USE OF PLASMOLYZED CELLS TO STUDY THE EFFECS OF HYPEROXIA ON RNA, DNA AND PROTEIN SYNTHESIS:

Plasmolyzed cells were used in order to make cells permeable to substrates so that synthesis of RNA, DNA and protein could all be compared in cells after oxygen exposure. Strain 161, a stringent organism was grown under nutritional conditions (all 20 amino acids present) which would prevent development of stringency from hyperbaric oxygen. Thus any effects seen in plasmolyzed cells, after oxygen exposure, should be attributable to "direct" effects on the particular synthesis process and not due to an indirect effect of stringency (inhibition by ppGpp).

DNA synthesis continued normally (within experimental variability) as shown in Table 9. This agrees with previous data which suggest there is little if any direct toxicity to the machinery for DNA synthesis. Reductions previously observed in intact cells measured while incubation in HPO, appeared after more than 30 min, and may have resulted from failure of reinitiation of chromosomal synthesis when the round of synthesis already initiated, was completed in hyperoxia.

Over the same period RNA synthesis was impaired as shown by the 1/3 lower synthesis capacity (Table 9). A similar reduction in ability to synthesize protein occurred (Table 9).

Impaired synthesis abilities for RNA and protein, shown by the plasmolyzed cells prepared from cultures exposed to 4.2 atm of oxygen for 30 min, may have resulted from a single damage site. This is possible because of the known interrelationships between RNA and protein synthesis. For example, damage to RNA synthesis from hyperoxia could have resulted in plasmolyzed cells which had decreased capacity to make not only RNA but also protein, because of defects in mRNA or on RNA.

TABLE 9

Protein, RNA and DNA synthesis capabilities of plasmolyzed cells, prepared after exposure of E. coli cells to 4.2 atm of oxygen for 30 min.

| Component | Experiment No. | CPM/mg protein per min. of incubation Control | $O_2$ Treated | Percentage Change |
|---|---|---|---|---|
| DNA | 1 | 2250 | 2130 | -5 |
| | 2 | 2020 | 1951 | -3 |
| | $\bar{x}$(avg) | 2135±163 | 2041±127 | -4 |
| RNA | 1 | 4170 | 2860 | -31 |
| | 2 | 4374 | 3697 | -15 |
| | 3 | 3073 | 2328 | -25 |
| | 4 | 3847 | 2624 | -32 |
| | $\bar{x}$(avg) | 3861±571 | 2877±588 | -26 |
| Protein | 1 | 220,130 | 146,160 | -34 |
| | 2 | 136,355 | 86,223 | -37 |
| | 3 | 191,047 | 128,062 | -33 |
| | $\bar{x}$(Avg) | 182,511±42,535 | 120,148±30,742 | -34 |

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Significant progress occurred in four areas: (1) Effect of hyperoxia on reverse glycolysis: We conclude that glucose protects Escherichia coli against hyperoxia better than other intermediates later in glycolysis and in the Krebs cycle but not because of a specific inhibition of reverse glycolysis. There was no significant impairment of the three enzymes of reverse glycolysis: fructose-1,6-diphosphatase, phosphoenolpyruvate synthase, or phosphoenolpyruvate carboxykinase. (2) Effect of hyperoxia

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on synthesis of RNA, DNA and pyridine nucleotide coenzymes: Evidence was obtained for direct inhibition of RNA synthesis in cells poisoned by hyperoxia, while protein synthesis was not directly affected. DNA synthesis is impaired but it is not proven whether this is direct or indirect. NAD and NADH were severely decreased in cells during poisoning by hyperoxia. This decrease was prevented by niacin, but not quinolinate which is consistent with poisoning of quinolinate phosphoribosyltransferase (QPT), as previously reported. PRPP does not protect against oxygen poisoning, PRPP synthetase is not affected by hyperoxia, but PRPP accumulates in oxygen poisoned cells which is in agreement with inhibition of QPT. (3) Relationship of thiamine to oxygen toxicity. Thiamine is protective for E. coli exposed to hyperoxia. Oxygen exposure appears to poison an enzyme required for synthesis of hydroxymethyl pyrimidine; other enzymes in the thiamine pathway are not affected.